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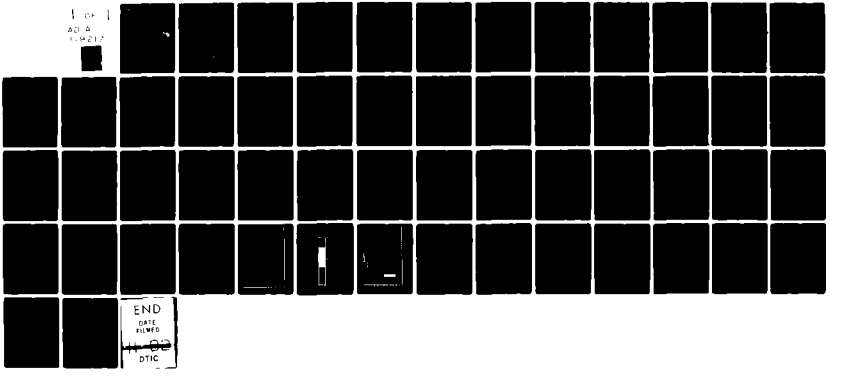

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The toxicity of acrylamide, n-methylacrylamide, and crotonamide as well as the organophosphates mipafox, leptophos, paraoxon, EPN, OMPA and DFP were studied in order to see whether or not in vivo-in vitro neurotoxicity correlations could be established. The in vitro systems employed were the mouse neuroblastoma cell line NIE-115 and the chick brain, either as cell aggregate cultures or organ culture.

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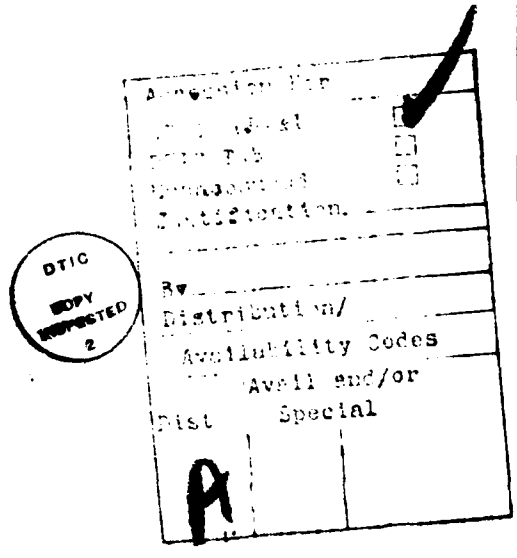
In both the neuroblastoma cell culture and chick brain cell/organ culture systems, acrylamide was the most toxic. The ranking of acrylamide, n-methylacrylamide and crotonamide paralleled the ranking reported in vivo. The end-points which showed this ranking included cell viability and neuron-specific enolase activity and aggregate formation by dissociated brain cells.

The organophosphate studies emphasized their effect on neurotoxic esterase activity. A model in vitro test has been developed for the evaluation of neurotoxic esterase effects. The test is based on the hen brain assay test developed by Johnson, as a predictor of delayed neuropathy.



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# IN VITRO STUDIES OF NEUROTOXIC SUBSTANCES: THE EFFECT OF ORGANOPHOSPHATES AND ACRYLAMIDES

Department of Biology
The Catholic University of America
Washington, D.C. 20064

Roland M. Nardone, Ph.D.
Jack Spiegel, Ph.D.
Albert Fedalei
David Krause
Rene M. Filipowski
J. Michael Mullins, Ph.D.

Controlling Office: USAF Office of Scientific Research NL
Bolling Air Force Base, D.C. 20332

CONTENTS

LIST OF FIGURES

LIST OF TABLES

## 1.1. Introduction:

The expanding use of *in vitro* systems for carcinogenesis, mutagenesis, and general toxicity testing attests to their potential and real value and ever-increasing role in monitoring the quality of our environment. Expectations associated with the use of *in vitro* systems include benefits in cost, time, and understanding (Nardone, 1980).

Despite the fact that mammalian cell cultures have been used for toxicity studies for about two decades, the field is still plagued by a number of inadequacies. These include, until 1975, a lack of any systematic studies aimed at the standardization and validation of *in vitro* test systems, and a failure to exploit the opportunity presented by the dramatic advances in tissue culture techniques and somatic cell genetics made during the past decade. These advances include some which offer great promise for the attainment of an expanded repertoire of cell types suitable for toxicity testing in general and for the testing of target specific substances, such as neurotoxic agents which may be of special interest to those responsible for monitoring the environment.

Among the neurotoxic agents are chemicals which are used in pest control, such as organophosphates and carbomates, as well as substances related to the manufacture of plastics and other materials. Casida *et al* (1976) have reported that over 200 million pounds of organophosphorus compounds are produced world-wide each year for biocidal purposes.

In view of the economic importance of these chemicals, continued attempts are being made to develop analogs which are less neutotoxic to mammals and, in the case of pesticides, more toxic to insects. Validated *in vitro* test systems developed specifically for the assessment of neurotoxicity in its

many forms could be useful aids in the evaluation of such substances.

Cell, tissue, and organ culture studies involving materials of neuronal origin have been employed since Harrison made his classical observations on nerve cells in tissue culture in 1907. Among the significant advances in neuronal cell cultures have been those which have involved the culture of continuously proliferating neuroblastoma and glioma cells which, in vitro, continue to manifest their differentiated properties. These include for neuroblastoma cell neurite formation, action potential generation, synapse formation, and specialized neurotransmission-related biochemical properties. Glioma cell in culture also show differentiated properties including the production of nervous system specific proteins and inducibility to catecholamine and cortisol.

Tissue and organ culture reaggregation studies with tissues such as brain have proved to be especially useful in the study of development, cell recognition, sorting and interconnection (Seeds, 1973). Organ cultures of spinal cord, dorsal ganglia, cerebrum, hippocampus, hypothalamus, and cerebellum have been successfully maintained in vitro. The success is illustrated by the work of Aparicio et al (1976), who observed that within 14 days after explantation, pieces of cerebellum showed "the basic organ features -- cortical layering, synapse formation, and myelination of axons".

The facility with which replicate cultures of genetically homogeneous cells can be maintained as monolayers, and the availability of clearly defined nervous system end-points in such cultures, suggest strongly that the development of model systems for the testing of neurotoxic substances should revolve strongly around monolayer cultures of replicating (transformed) cells. These should be complemented by the use of primary cell cultures and brain aggregate and organ culture systems for those studies which may require end-

points not manifested by monolayer cultures.

Research in the area of in vitro mutagenesis and carcinogenesis has emphasized the need for testing to involve batteries of tests with overlapping and complementary end-points (Nardone, 1981). Indeed, that view is a basic underpinning of this research program. Hence, in addition to a variety of cellular targets (embryonic brain cells and organ culture, established cell lines of neuronal and non-neuronal origin, and primary spinal cord cultures), a matrix of biochemical, cell viability, and cell behavior tests relating to neuronal and non-neuronal function are used to test the xenobiotics for their toxicity. Among the end-points selected for use are the following: neuron-specific enolase, total enolase, neurotoxic esterase, acetylcholine esterase, choline acetyltransferase, and tyrosine hydroxilase. Other end-points involve acetylcholine receptors, biosynthesis rates, cell aggregation, cell viability, and plating efficiency.

The development of a model system for testing xenobiotics requires extensive validation. For such purposes related and unrelated chemicals whose in vivo toxicity ranking is known, are employed. Ideally, the specific mode of action of the test chemicals should also be known. Acrylamides and organophosphates satisfy these criteria (at least in part) and have been selected as the test chemicals used in the initial validation. Because of the greater economic importance of the organophosphates and the larger data base regarding their diverse biological effects, they constitute the chemicals of choice for most of the research which will be undertaken for this project.

The organophosphate (OP) compounds represent a diverse group of chemicals widely used as pesticides, lubricants, hydraulic fluids, plasticisers, and flame retardants (Johnson, 1980). In view of the widespread distribution of these compounds and the diversity of their applications, it is essential to

health and safety that the toxicity of these compounds be understood and anticipated prior to full scale production of new OPs.

The OP compounds produce two distinct and unrelated types of toxicity. The first is an acute toxicity which occurs within minutes following exposure. It is characterized by excessive cholinergic stimulation. This results from the binding of an OP to acetylcholinesterase (AchE) and subsequent inactivation of this neuronal enzyme. Without active AchE present at post-synaptic membrane surfaces, the neurotransmitter acetylcholine is not degraded and thereby accumulates. This leads to excessive stimulation of the muscarinic and nicotinic receptors within the nervous system. Some symptoms include excessive lacrimation, salivation, sweating, involuntary muscle contraction urination, defecation, and weakness. Death usually results from asphyxiation due to excessive bronchocontraction, bronchosecretion, and paralysis of the respiratory muscles (Murphy, 1980). The mechanisms of this type of toxicity is well understood. High risk populations can be monitored for exposure by assessing RBC and plasma cholinesterase, which can be done by the rapid and sensitive method developed by Ellman et al (1961)

The second type of toxicity induced by OP esters is a delayed neurotoxicity syndrome. It is characterized clinically by ataxia and paralysis beginning in the distal portion of the hind limbs and advancing proximally. The forelimbs become involved in severe cases. There is a latent period of 6 to 14 days between exposure to the OP and onset of clinical symptoms. The term organophosphorus ester induced delayed neurotoxicity (OPIDN) is now widely accepted to describe this syndrome (Abou-Donia, 1981). There is wide variation in the sensitivity of species to OPIDN, with man, chicken and cats being very sensitive while rodents are very resistant (Abou-Donia, 1981).

In addition, children and young animals are much less sensitive to OPIDN than are adults and mature animals. Recovery from OPIDN is extremely slow and often incomplete (Johnson, 1980).

The histopathology of OPIDN has been extensively investigated by Bouldin and Cavanagh (1979a,b) using teased fiber preparations of nerve and electron microscopy. There is a selective involvement of the longest and largest diameter myelinated axons of the central and peripheral nervous system. In the early stages of OPIDN distal, but not terminal nerve fiber varicosities, are observed. Demyelination then occurs around the swelling followed by degeneration of the distal portion of the axon. This OP induced focal degeneration is thought to produce a chemical transection of the axon which then precipitates a wallerian degeneration of the distal terminus of the axon. Demyelination follows, but it is important to note that this is secondary to the axonal degeneration. This entire process proceeds proximally toward the nerve cell body in a saltatory fashion producing an incremental degeneration of the distal axon.

Abou-Donia (1981) has reported that 40,000 cases of delayed neurotoxicity in man have been documented. The first cases of OPIDN were reported to have occured at the end of the nineteenth century in tuberculosis patients treated with phospho-creasote. In 1930 a massive outbreak of OPIDN affecting up to 20,000 persons occurred as the result of the ingestion of an illegal liquor substitute known as Jamaica ginger containing tri-orthocresylphosphate (TOCP). Many other outbreaks of OPIDN have been recorded since 1930, the most recent of which occurred in 1975 and affected scores of Egyptian farmers and domestic livestock (Iker, 1982). The OP insecticide lepthophos (also known as phosnel) which was unregistered in the United States, was responsible for this disaster. In 1976, 500 Pakistani workers were poisoned by mis-

handling the OP insecticide malathion. Five fatalities occurred. The World Health Organization (WHO) reports that in 1972, 500,000 persons were poisoned by pesticides exported to third world nations in large quantitites. These pesticides are often applied excessively and improperly by untrained or illiterate workers (Iker, 1982).

Johnson (1968) found that a neurotoxic dose of $^{32}$P-labeled diisopropyl fluorophosphate (DFP) labeled hen brain homogenates in vitro. Preincubation of the homogenate with a non-neurotoxic OP reduced labeling by $^{32}$P-DFP. Preincubation with a neurotoxic OP led to further reduction in label by $^{32}$P-DFP. Johnson proposed that the fraction of labeling reduced by a neurotoxic OP but not by the non-neurotoxic OP might represent a biochemical target site for the action of neurotoxic OPs. Johnson (1970) showed a positive correlation between phosphorylation of this brain fraction and clinical development of OPIDN in hens. This fraction was found to be capable of hydrolyzing the ester phenyl phenylacetate and was subsequently named neurotoxic esterase (NTE). Certain carbomates and sulphonates in addition to phosphates were found to bind and inhibit NTE but did not cause delayed neuropathy. In vivo administration of sulphonates or carbomates actually provided protection to hens against subsequent challenge doses of neurotoxic OPs. This protection lasted until 70% of the NTE again became available for phosphorylation. Simple inactivation of NTE is not responsible for OPIDN but the chemical nature of the bound compound is crucial. Johnson (1974) found that phosphinates behaved like carbamates and sulphonates against NTE, inhibiting it but not causing OPIDN. It is proposed that the development of OPIDN requires not only phosphorylation of NTE, but subsequent hydrolysis of a phosphoryl ester bond to leave a charged monosubstituted phosphoric acid group attached to the enzyme. This charged complex is then thought to be

responsible for disrupting the normal function of the anon causing neuropathy. Further evidence for this process emerged in 1979 when the kinetics of NTE aging was studied (Clothier and Johnson, 1979). Aging occurred with a half life of 2-4 minutes after reactivation of NTE with KF was impossible. The mechanism of aging of NTE seems to be distinctly different from the better characterized dealkylation aging of the cholinesterase. Much is still unknown about NTE and OPIDN. The rate and sequence of events between phosphorylation and aging of NTE, and the production of axonal degeneration is completely unknown. The physiological significance of NTE is a mystery as well since its activity within the neuron does not seem to be essential for cell survival or function.

Acrylamides represent another class of neurotoxic chemicals which are widely used and diverse in their application. Since the early 1950's acrylamide has been used as a flocculator to strengthen paper and cardboard, as a grouting agent, as a separating menstrum, and for many other purposes (McCollister et al, 1964). In 1972, 35 million pounds of the polymer and 50 million pounds of the monomer were produced in the United States (Spencer and Schaumberg, 1974).

Soon after its introduction, the neurotoxicity of the monomer became recognized among industrial workers (Fujita et al 1960). Low exposure led to drowsiness and a lack of concentration while higher exposure was accompanied by distal numbness and weakness.

In animals, acrylamide caused ataxia and seizures. Fullerton and Barnes (1966) reported that acrylamide causes peripheral nerve distal neuropathy. Such a dying-back pattern of damage is similar to that associated with organophosphorus-type degeneration.

The toxicity of acrylamide can be reduced by molecular changes. Hence,

while the $LD_{50}$ for acrylamide (AA) dosed rats is 1.5 mmol/kg, the $LD_{50}$ for N-methylacrylamide (NMA) and crotonamide (CA) are 5.6 and 32 mmol/kg, respectively (Hashimoto et al, 1981). The toxicity may stem from several factors, most notably the inhibition of neuron specific enolase. a nerve-specific form of enolase, and other glycolytic enzymes, such as glyceraldehyde-3-phosphate dehydrogenase and phosphofructokinase (Howland et al, 1980a,b).

Hence, the organophosphates and the acrylamides represent good candidates to serve as model compounds for the development and validation of an in vitro model for neurotoxicity. Both are neurotoxic. The classes include analogs whose in vivo neurotoxicity ranking is known and differs from compound to compound. The mechanism of toxicity is understood, at least in part. Thus, molecular targets have been identified.

1.2. Objectives:

The objectives for the first year of this research program are as follows:

1. To establish the cell and organ culture systems to be used for testing. For the first year, these include a chick brain cortex organ culture, an established cell line with neuronal properties (NIE-115) and a non-neuronal cell line (Strain L).

2. To establish the assays for monitoring neuronal and non-neuronal end-points which will yield information regarding neuronal and general cytotoxicity, respectively.

The neuronal end-points which have been selected include neurotoxic esterase, neuron-specific enolase, and acetylcholinesterase, and microtubule formation in neurites. The non-neuronal end-points include DNA, RNA, and protein synthesis, cell viability, plating efficiency, enolase activity, and esterase activity.

3. To determine the relative toxicity of acrylamides and several organophosphates in these systems.

2.1 Methods:

Cell Aggregation and Cortex Organ Culture:

Cerebral cortices were aseptically dissected from the corpus striatum of 10-day white leghorn chick embryos; staged according to Hamburger and Hamilton (1951). The cortices were freed from their meninges and washed in sterile Tyrode's solution. For aggregation studies, the cortices were washed 3 times in calcium and magnesium-free Tyrode's solution (CHF), and prepared for dissociation according to the procedure of Moscona (1961). Briefly, this involves incubation in CMF at 37°C for 10 minutes, followed by incubation in 0.67% trypsin (Grand Island) solution at 37°C for 20 minutes in a 5% $CO_2$ - 95% air atmosphere. After 3 washes with CMF control culture medium (Medium 199, supplemented with 1% L-glutamine, 50 units/ml each of penicillin and streptomycin, and 25 µg/ml DNAse I) were added, and the tissue was mechanically dissociated into single viable cells by repeated flushing through a fine-bone pasteur pipette. Approximately 1 x $10^7$ cells (cells from 3 cortices) were dispensed into 3 ml of the control or test medium contained within a 25 ml erlenmeyer flask. The flasks were sealed in a 5% $CO_2$ - 95% air environment and incubated at 37.5°C in a rotary shaker (3/4 inch orbit of rotation) at 70 RPM. Organ cultures of 10-day embryonic chick cortex were similarly incubated on the rotary shaker in 3 ml of medium minus the DNAse.

Acetylcholinesterase Assay:

Cortex organ cultures or aggregates (pelleted) were homogenized in 0.1 M phosphase buffer (pH 8.0), and assayed for acetylcholinesterase activity using the colorimetric method of Ellman et al (1961). Using acetylthiocholine as the substrate, enzymatic hydrolysis yields thiocholine, which forms a color complex with 5, 5' Dilhiobis-2-nitrobenzoic acid. Specific activity of acetylcholinesterase is expressed as nanomoles of acetylthiocholine hydrolyzed per

minute per mg protein or as nonomoles of acetylthiocholine hydrolyzed per minute per cortex (for organ culture) or per cortex equivalent (for aggregate cell culture). True acetylcholeresterase activity is distinguished from total cholinesterase activity by the addition of 80 μM tetraisopropylpyrophosphoramide, a selective irreversible inhibitor of non-specific cholinesterase. Protein determinations were made by the method of Lowry et al (1951).

Morphological Appraisals of Cortices:

Cortices were fixed using 3% paraformaldehyde -- 2.8% glutaraldehyde in 0.05 M phosphate buffer pH 7.5, post-fixed for 3 hours in 1% osmium, dehydrated through alcohol and acetone, and embedded in Spurr. One micron sections were stained with azure-methylene blue. Thin sections were prepared as follows: hardened blocks were trimmed and sectioned using a diamond knife to obtain thin sections. Thin sections were stained with both lead and uranium, and examined at 60 kV in a Zeiss Electron Microscope. For light micoscopy, 1 μm sections were cut using glass knives and stained with toluidine blue.

Electron Microscopy of Neuroblastoma Cells:

In order to preserve the characteristic morphology of the neuroblastoma displayed in monolayer, cultures were fixed initially in situ (Goldman, 1972). Initial fixation was in 2% glutaraldehyde, 0.1 m cacodylate buffer, pH 7.2. After approximately 20 minutes in this fixative the cells were gently scraped from the growth surface using a rubber policeman. Fixation was allowed to continue in glutaraldehyde for a total of 45 minutes. At this point cells were pelleted and processed as a pellet through subsequent procedures. Post-fixation was in 1% $OsO_4$ in 0.1 M cacodylate buffer, pH 7.2 for 30 minutes. Cells were stained en bloc with 0.5% aqueous uranyl acetate, dehydrated through an acetone series and embedded in Spurr's epoxy resin mixture (Spurr, 1969). Polymerized blocks were sectioned using a DuPont diamond knife and a Reichart

ultramicrotome. Sections were stained with lead citrate (Reynolds, 1963) and examined at 60 KV using a Zeiss Electron Microscope.

Cell Culture Methods, Cytodifferentiation and Neurotoxic Esterase (NTE) Assays:

Cell culture methods described below for NTE studies were also employed for studies on acetylcholinesterase and neuron-specific enolase inhibition by acrylamides.

Intact cultures of differentiated neuroblastoma C-1300 clone NIE-115 (Amano et al, 1972) are used in place of whole animals. Cell culture homogenates are used in place of hen brain homogenates. Cell line NIE-115 was kindly provided by M. Nirenberg of the NIH. Cell cultures were grown as monolayers in Corning 75 $cm^2$ polystyrene flasks (Corning Glass Works, Corning, N.Y.), with Dulbecco's modification of Eagle's minimum essential medium (DMEM), obtained from Flow Laboratories (McLean, Va.). Media was supplemented to a 10% concentration with newborn bovine serum (Flow) and buffered with 3700 mg/L of sodium bicarbonate. Cultures were maintained in an environment of 10% $CO_2$ - 90% air at 37°C (Air Products, Hyattsville, Md.). These cells possess the capability to differentiate morphologically, biochemically, and electrophysiologically in the presence of cAMP. Cell cultures were differentiated by withdrawal of serum and addition of 0.5 mM dibutyryl adenosine 3', 5'-cyclic monophosphate (cAMP) from Sigma when cells had reached stationary phase. All OP stocks were prepared at a 20 mM concentration in DMSO (Fisher Scientific Company) and added to whole cultures or homogenates as indicated.

The following organophosphate compounds were obtained from the Environmental Protection Agency Analytical Reference Standards Repository, Raleigh, N.C.: O-methyl O-4 bromo-2, 5-dichlorophenyl phenylphosphorothioate (leptophos), O-ethyl O-4-nitrophenyl phenylphosphorothiate (EPN), actomethyl pyrophosphoramide (OMPA), O, O-diethyl O-P-nitrophenyl phosphate (paraoxon). All reference standards were greater than 98% pure. N, N diisopropylphosphorodiamidic

flouride (mipafox) and phenyl valerate were kindly provided by M.B. Abou-Donia, Duke University. DFP was obtained from Sigma. Leptophos-oxon was also obtained from the EPA. After exposure of cultures to OP's in situ the cells were rinsed in saline, scraped, homogenized in a 7 ml Wheaton Dounce for 20 strokes, and assayed according to the method of Johnson (1977) for NTE with necessary modifications. This method is a differential assay requiring the two OP's paraoxon and mipafox to define the amount of NTE present. Paraoxon (50 μM) is added to all test samples except for a control to inactivate irrelevant esterase activity. Mipafox (200 μM) is then added to a sample. Inhibition of esterase activity beyond that inhibited by the paraoxon is NTE. Any inhibition of a test OP beyond that due to the paraoxon is defined as inhibition of NTE. Johnson (1975) found phenyl valerate to be a more specific and sensitive substrate than phenyl phenylacetate for measuring NTE so this is now the substrate of choice. Esterase activity is measured spectophtometrically at a wavelength of 510 mM. Phenol red is found by the hydrolysis of the substrate, hence the more activity the higher the O.D. 510.

2.2 Experimental Results:

2.2.1 Dose Response to Acrylamide: Chick Brain:

Dissociated 10-day embryonic chick cerebral cortex (C x $C^{10}$) cells were aggregated for 24 hours in serum-free control medium and compared to C x $C^{10}$ cells aggregated in 1, 3, 5, 10 and 15 mM acrylamide. One mM acrylamide had no effect on the size or morphology of 24 hour aggregates. Three and five mM acrylamide affected a slight reduction in aggregate size, but the aggregate morphology was still compact and similar to the control aggregate. Eight mM acrylamide elicited a definite effect upon aggregate morphology. The aggregates were smaller, less compact, more flattened, and much more irregular in shape. This partial inhibition of aggregate formation was enhanced with 10 mM acrylamide exposure. Cultures aggregated in the presence of 10 mM acrylamide were markedly smaller; with irregular aggregate contours or shapes. Fifteen mM acryalmide caused a complete inhibition of cellular aggregation.

2.2.2 Kinetics of Aggregation Inhibition by 10 mM Acrylamide:

The initial 2 hours of aggregation were similar to control cultures. The first indication of an effect of 10 mM acrylamide on C x $C^{10}$ cell aggregation was at 3-4 hours; taking the form of aggregate clusters with slightly less regular contours. The toxic response of 10 mM acrylamide was markedly evident by approximately 6 hours of aggregation. By this time, control aggregates were initiating the process of compaction into definitive spheroid shapes with regular contours. By contrast, aggregate clusters in the presence of 10 mM acrylamide remained smaller and did not become compact.

In control cultures, the time period from 6 - 10 hours of aggregation involved a period of aggregate growth (primarily via secondary fusions of primary aggregates followed by compaction). Aggregates in the presence of 10

mM acrylamide also enlarged during this time, but the aggregate fusions were more "agglutination-like"and were not followed by condensation into compact aggregate forms.

During the time period from 10 - 24 hours of aggregation, control aggregates continued to secondarily fuse and compact into their definitve spheroid aggregate shape. Aggregates cultured in the presence of 10 mM acrylamide deteriorated from their condition at 10 hours of culture, becoming the characteristic small, loose, flattened, irregular shaped cluster aggregates by 24 hours.

To test if there was a particular time during early aggregation which was critically sensitive to the toxic effects of acrylamide, I administered acrylamide (to a final concentration of 10 mM) to cultures at various time points (2, 3, 4, 7, 8 1/2, and 10 hours) after the onset of aggregation in control medium. Regardless the time of acrylamide administration, the aggregates at 24 hours of culture were grossly affected in both size and appearance.

2.2.3 Aggregation of C x $C^{10}$ Cells Pre-exposed to Acrylamide as Organ Cultures:

Taking advantage of the thin sheetlike nature of the embryonic chick cortex, I exposed the intact cortices to 10 mM acrylamide for 10 - 12 hours as rotating organ cultures. The gross appearance of these acrylamide exposed cortices indicated a toxic response compared to the corresponding control cortices cultured for the same period in serum-free medium. The acrylamide exposed cortices were beginning to fragment, and there was a slight turbidity due to the presence of free cells in the culture medium. The acrylamide exposed cortices were trypsin dissociated, and the aggregates formed by these cells in serum-free control medium were monitored after 24 hours of aggregate culture. This acrylamide exposure prior to tissue dissociation did

affect the appearance of the 24 hour aggregates. These aggregates were smaller than their corresponding control aggregates and not quite as compact. However, their appearance was strickingly improved over freshly dissociated cortex cells aggregated 24 hours in the continuous presence of 10 mM acrylamide. The aggregation of the control cultures was not affected by the 10 - 12 hours prior organ culture exposure in serum-free culture medium.

The aggregation of pre-exposed (10 - 12 hours in 10 mM acrylamide as organ cultures) was monitored at time points prior to 24 hours. The first stages (first 2 hours) were similar to the control situation. By 3 hours and continuing through 6 -7 hours, the acrylamide pre-exposed cells remained small loose cluster aggregates with some secondary fusions, while the control cultures continued to grow and began to become compact. By 12 hours of culture, however, the acrylamide pre-exposed cultures appeared to be recovering from this toxic effect, and began to compact. This apparent recovery continued to the 24 hour termination time point.

The organ culture pre-response manipulation was exploited to determine the minimum pre-exposure time to acrylamide required to elicit an effect upon subsequent aggregation of the dissociated cells in serum-free control medium. Thus, corticeswere pre-exposed to 10 mM acrylamide for 2, 5, or 8 hours, and then processed for aggregation. The resultant 24 hour aggregates demonstrated a graded response to the prior acrylamide exposure, ranging from no effect upon aggregation with the 2 hour pre-exposure to a moderate effect after 5 hours pre-exposure to a definite effect following the 8 hour pre-exposure.

2.2.4 Effect of N-Methyl Acrylamide on C x $C^{10}$ Cell Aggregates:

Freshly dissociated C x $C^{10}$ cells were aggregated 24 hours in the presence of 20 mM N-methylacrylamide. The resultant 24 hour aggregate demon-

strated a slightly affected appearance. They were somewhat smaller (reminiscent of 5 mM acrylamide) and they were beginning to show signs of the more jagged aggregate contours characteristic of 8 and 10 mM acrylamide exposure. The aggregation of C x $C^{10}$ cells in the presence of 20 mM N-methyl acrylamide was followed at early stages of aggregation (2, 3, 6, 7, 8, and 9 hours). There was no appreciable difference in appearance of these aggregates to corresponding controls in serum-free medium. Apparently, the slight inhibitory effect of N-methyl acrylamide on 24 hour aggregation begins later in the aggregation process, and thus has a different type of kinetics than 10 mM acrylamide inhibition.

Cortices were pre-exposed to 20 mM N-methyl acrylamide as organ cultures for 12 hours, and then dissociated and aggregated in control medium. The pre-exposed cells did demonstrate some noticeable differences from controls during the time points from 3 to 8 hours of aggregation. Similar to acrylamide, these aggregates were loose, flat and had less regular contours than control aggregates. However, these aggregates seemed to undergo more early secondary fusions causing these early aggregate clusters to be larger than the corresponding controls. While still somewhat larger and looser, these pre-exposed aggregates began to become compact by 9 - 11 hours of aggregation, and by the 24 hour termination point they looked similar to the controls.

2.2.5 Acetylcholinesterase Levels in C x $C^{10}$ Cultures Exposed to Acrylamide and N-methylacrylamide:

Acetylcholinesterase (AchE) levels were assayed both in aggregation cultures and in intact C x $C^{10}$ cortices maintained as organ cultures. AchE levels were monitored at 2 time points following exposure to acrylamide: 8 hours and 24 hours. AchE specific activities are expressed both in terms of enzyme rate per mg of protein and enzyme rate per cortex used (cortex equiva-

lent in the case of aggregates). In light of the cummulative error resulting from the vagaries of tissue dissection, dispensing equal numbers of cells in the case of aggregate studies, and the biochemical assays, I submit that percentage differences between control and experimental cultures that are less than ≈10% are probably of questionable significance.

Table I indicates the mg/protein/cortex, nmoles/min/cortex (cortex equivalent), and nmoles/min.mg protein AchE specific activities for non-cultured 10-day embryonic chick cortices. These indices for the non-cultured cortices represent the baseline values against which percentage changes are calculated for the other experimental cultures listed in this table.

C x $C^{10}$ cortices maintained as organ cultures for 8 hours in control medium demonstrate a somewhat reduced mg/cortex/ content (18%↓ in response to the culture exposure in serum-free medium. A significant proportion of this reduced protein content represents generalized tissue degeneration as AchE specific activity in terms of nmoles/min/cortex is reduced ≈11%. However, the effects of the culture conditions are somewhat more pronounced in reducing the total protein content than the relative amount of AchE as seen in the slight increase in AchE specific activity expressed in terms of nmoles/min/mg protein. It should be noted that the level of tissue decay represented by this protein decline is not sufficient to affect the size or morphology of 24 hour aggregates formed when these pre-incubated cortici are dissociated and the cells are reaggregated.

Exposure of the 8 hour organ cultures to 10 mM acrylamide caused a marked decrease in protein content (3%↓). This reduced protein content is <u>not</u> reflected proportionately in the levels of AchE activity. The nmoles/min/cortex value is down only 8% indicating that the depressed protein content has

not yet led to generalized degeneration. AchE specific activity expressed in terms of mg protein demonstrates a 45% increase, indicating that the AchE enzyme is not a target of the reduced protein levels in response to acrylamide.

Table II examines the same data for acrylamide exposure, however, now expressed relative to its corresponding control culture rather than to the non-cultured cortex starting material. Expressed this way, there is no significant change in AchE enzyme activity per cortex. The AchE activity per mg protein is elevated (28%) in proportion to the reduced protein content (24%). Indicating again, that the toxic effect of 10 mM acrylamide on total cellular protein does not encompass a corresponding decline in AchE levels; and certainly not a specific inhibition of AchE activity.

When the control organ cultures are maintained in serum-free medium for 24 hours, Table I again indicates that such culture conditions cause a decrease in total protein content (28%↓). This reduced protein content probably represents generalized tissue and cell decay as corroborated by the proportionate decline (21%) in AchE activity per cortex used. Correspondingly, the AchE activity per mg protein remains essentially unchanged.

C x $C^{10}$ cortices maintained in serum-free medium containing 10 mM acrylamide exhibited a massive decrease in protein content (75%↓). A considerable portion of this protein decrease was translated into tissue distruction as evidenced by the 49% decline in AchE activity per cortex used. This correlates well with the poor gross appearance of these cultures (fragmented, loose cortices with free cells in the medium). While there was considerable tissue damage, the loss of AchE activity did not parallel the large magnitude of the protein decrease. This is similar to the effect of acrylamide on 8 hour cultures. As a result, the nmoles/min/mg protein specific

activity rose pricipitously. When the cultures of 24 hour cortices in 10 mM acrylamide are compared to their corresponding control (Table II), there was a 68% drop in protein content/cortex, corresponding to a 36% decline in AchE activity per cortex used. Again, the loss of AchE activity did not keep pace with protein loss yielding a large increase in AchE activity per mg protein, further evidence that AchE is not a specific target for acrylamide inhibition in this system.

When C x $C^{10}$ cortices are trypsin dissociated and aggregated for 8 hours in serum-free medium, there is a large decrease in relative protein content (57%↓) compared to the equivalent amount of initial cortex material in situ. This is not unreasonable considering the drastic events of neural tissue dissociation. There was a proportionate 55% decline in AchE activity per cortex equivalent corresponding to this dissociation-related tissue damage.

Aggregation of the dissociated cells for 8 hours in 10 mM acrylamide again caused an enhanced decrease in protein control per cortex equivalent (73%↓). Table I also indicates that this additional protein loss was not yet translated into additional gross tissue destruction; i.e., AchE-activity is down only slightly more than 8 hour control cultures. Similar to the case with cortex organ cultures, the acrylamide induced decline in protein content was not paralleled by a proportioanl decrease in AchE activity. Thus, the 60% increase in AchE activity per mg protein.

When compared to their 8 hour control aggregates (Table II), we again see the acrylamide induced toxicity on protein content (38%↓) which is not correlated with a corresponding decline in Ache activity (i.e., AchE activity per mg protein is up the proportionate 34% and SchE activity per cortex equivalent remain relatively unchanged).

When control aggregates are maintained in serum-free medium for 24 hours, the number do not change dramatically from the situation in 8 hour control aggregate cultures. There is a slight drop in AchE specific activation, which probably corresponds to the additional culture time in the minimal serum-free medium.

Aggregation for 24 hours in the presence of 10 mM acrylamide exacerbates this general decline. Protein content is again dramatically decreased by the acrylamide toxicity (89% compared to the original starting tissue and 66% compared to the corresponding 24 hour control aggregates). The aggregate tissue atrophy seen in the gross morphology of the aggregates is again correlated with the decline in AchE activity per cortex equivalent compared to the control aggregates. However, AchE again does not appear to be the primary target of acrylamide toxicity for the AchE activity per mg protein is actually 44% higher than the corresponding control. Again, this reflects the fact that the protein content is going down faster in response to acrylamide than tissue destruction is eliminating AchE.

Like acrylamide, N-methyl acrylamide is toxic and does not appear to act directly upon AchE. The N-methyl acrylamide is of course less toxic, with twice the dosage having a less dramatic and more evenhanded general toxic effect upon the cultures.

Finally, there is the direct evidence that acrylamide does not specifically inhibit AchE activity. When acrylamide was added to a final concentration of 100 mM to the reaction mixture of AchE assay for non-cultured cortices, it did <u>not</u> change the slope of the rate reaction curve.

Table I. Effect of acrylamide on protein content and acetylcholinesterase activity by chick brain organ culture.

| Specimen | Mg protein/cortex (equivalent) | nmoles/min/cortex (equivalent) | nmoles/min/mg protein |
|---|---|---|---|
| Non-cultured Cortices | 0.097 (100%) | 5.3 (100%) | 54.1 (100%) |
| 8 hr Control Cortices | 18%↓ | 11%↓ | 12%↑ |
| 8 hr Cortices in 10 mM Acrylamide | 37%↓ | 8%↓ | 45%↑ |
| 24 hr Cont Cortices | 24%↓ | 21%↓ | 4%↑ |
| 24 hr Cortices in 10 mM Acrylamide | 75%↓ | 49%↓ | 132%↑ |
| 8 hr Control Aggregates | 57%↓ | 55%↓ | 20%↑ |
| 8 hr Aggregates in 10 mM Acrylamide | 73%↓ | 58%↓ | 60%↑ |
| 24 hr Control Aggregates | 60%↓ | 64%↓ | 10%↓ |
| 24 hr Aggregates in 10 mM Acrylamide | 84%↓ | 84%↓ | 29%↑ |

Table II. Effect of acrylamide and n-methylacrylamide on protein content and acetylcholinesterase activity of aggregates of brain cells.

| Treated Culture Compared to Its Respective Control | Mg Protein / Cortex (equiv.) | nmoles/min/cortex (equiv.) | nmoles/min/mg/protein |
|---|---|---|---|
| 8 hr Cortices in 10 mM Acrylamide | 24%↓ | 4%↑ | 28%↑ |
| 24 hr Cortices in 10 mM Acrylamide | 68%↓ | 36%↓ | 124%↑ |
| 8 hr Aggregation in 10 mM Acrylamide | 38%↓ | 6%↓ | 34%↑ |
| 24 hr Aggregation in 10 mM Acrylamide | 66%↓ | 47%↓ | 44%↑ |
| 24 hr cortices in 20 mM N-methyl acrylamide | 32%↓ | 37%↓ | 9%↓ |
| 24 hr Aggregation in 20 mM N-methyl acrylamide | 34%↓ | 34%↓ | 4%↓ |

2.2.6. Effect of Acrylamide, N-Methylacrylamide and Crotonamide on Viability and Plating Efficiency of NIE-115 Cells:

The neuroblastoma cell line NIE-115 was studied in a proliferating, non-neuronal stage and after differentiation by cAMP into a neuron-like stage of differentiation.

The $LD_{50}$ dose for proliferating cells, as determined by trypan blue exclusion, was 2.3 mM for acrylamide (AA) and 50 and 75 mM for N-methylacrylamide (NMA) and crotonamide (CA), respectively. For differentiated cells the $LD_{50}$ doses were 1.0 (AA), 35 (NMA), and 50 nM (CA).

The $ID_{50}$ doses for plating efficiency of proliferating neuroblastoma cells were 1.0 (AA), 25 (NMA), and 50 mM (CA) while the $ID_{50}$ doses for cell proliferation were 2.0 (AA), 20 (NMA) and 50 nM (CA).

Hence, it can be seen that the toxicity of the three acrylamides parallel the reported in vivo ranking, with plating efficiency being more sensitive to inhibition than cell proliferation or cell viability. Furthermore, the differentiated cells were more sensitive to each of the acrylamides than the undifferentiated cells. The linearity of the dose-response curves is typified by that for plating efficiency shown in Fig. 1.

2.2.7. Effect of AA, NMA, and CA on Macromolecular Synthesis:

The effect of AA, NMA, and CA on protein, DNA, and RNA content of differentiated NIE-115 cells and log phase cells, is shown in Tables 3 and 4 while Table 5 shows the effect of these chemicals on macromolecular synthesis rates. Low doses of AA in the range of 0.01 mM to 1.0 mM had no effect on protein and RNA content, but did reduce the DNA content, when all cells (trypan blue positive and negative) are included in the analysis and calculation. If one takes into account only the viable cells, then there was a

slight increase in the content of macromolecules as the dose of AA increased. N-methylacrylamide at dose of 10 to 40 mM caused an increase in protein content and in RNA content, while also inducing a decline in the DNA content. These increases were in the range of 10-25%. CA caused similar effects at slightly higher doses (from 30 to 50 mM). These effects induced by NMA and CA are consistent with the morphological differenttiation observed when similar doses were administered to log phase cultures in proliferation studies.

Control 8 day differentiated cells had 713 μg protein, 202 μg RNA, and 4.6 μg DNA per $10^6$ cells. This was a 27% increase over log phase cells in the amount of protein and a 13% increase in the RNA content. At the same time the DNA content of the cells dropped by 44%. Differentiated NIE-115 neuroblastoma cells were not affected by the xenobiotics except at doses which caused greater than 50% lethality. Then, only the protein levels were lowered by about 20% (Table 3).

Tables 4 and 5 list the protein, RNA, and DNA synthesis data for log phase and differentiated cells, respectively. Log phase NIE-115 neuroblastoma cells synthesized protein at a rate of 54% higher than that of differentiated cells. Log phase cells also had a 160% higher RNA synthesis rate and a 1790% higher DNA synthesis rate, than did differentiated cells.

Log phase cultures treated with AA did not show any inhibition of macromolecular synthesis until doses which caused greater than 50% lethality were administered. Doses of 2.0 mM AA reduced the protein, RNA, and DNA synthesis rates by only 27%, 27% and 47%, respectively. Both NMA and CA treated cultures did not show any inhibitory effects on protein and RNA synthesis, at doses below 40 mM and 75 mM, respectively. N-methylacrylamide, at dose of 5.0, 10, and 20 mM caused increases in the protein and RNA synthesis rates of log

phase cells, while causing a simultaneous decrease in the DNA synthesis rate. This effect was also seen with doses of CA ranging from 20 to 50 mM. These observations are consistent with the morphological differentiation and reduced growth rates noted earlier, which suggest that moderate doses of NMA and CA induce log phase neuroblastoma (NIE-115) cells to cease proliferating and to begin differentiating.

The protein, RNA, and DNA synthesis rates of differentiated NIE-115 neuroblastoma cells were slightly inhibited by doses which were below the $LD_{50}$ dose (1.0 mM AA, 35 mM NMA, and 50 mM CA). At doses of 1.0 mM AA the protein synthesis rate was reduced by only 12% and RNA synthesis was reduced by 25%. N-methylacrylamide treated cultures showed a 49% reduction in RNA synthesis at 30 mM concentration but only a 12% reduction in the protein synthesis. Crotonamide, at doses of 40 mM inhibited protein synthesis by 11% and RNA synthesis by 39%.

2.2.8. Effect of Acrylamides on Acetylcholinesterase (AchE) activity:

Undifferentiated log phase neuroblastoma cells express small amounts of AchE activity (Augusti-Tocco and Sato, 1969; Anano et al, 1972). Upon differentiation as much as 20 to 30-fold increase is sometimes noted (Blume et al, 1970; Schubert et al, 1971; Kates et al, 1961; Harkins et al, 1972; Prasad and Vernadakis, 1972). In the research reported herein a ten-fold increase (82.1 nmol product formed/min/mg protein to 865 nmol product formed/min/mg protein) in AchE activity was observed when log phase cultures were induced to differentiate with serum-free medium and 0.5 mM dibutyryl cAMP.

Non-differentiated and differentiated cultures were treated with various doses of AA, NMA, and CA under standardized conditions. Acetylcholinesterase activity was determined by the colorimetric procedure of Ellman et al (1961).

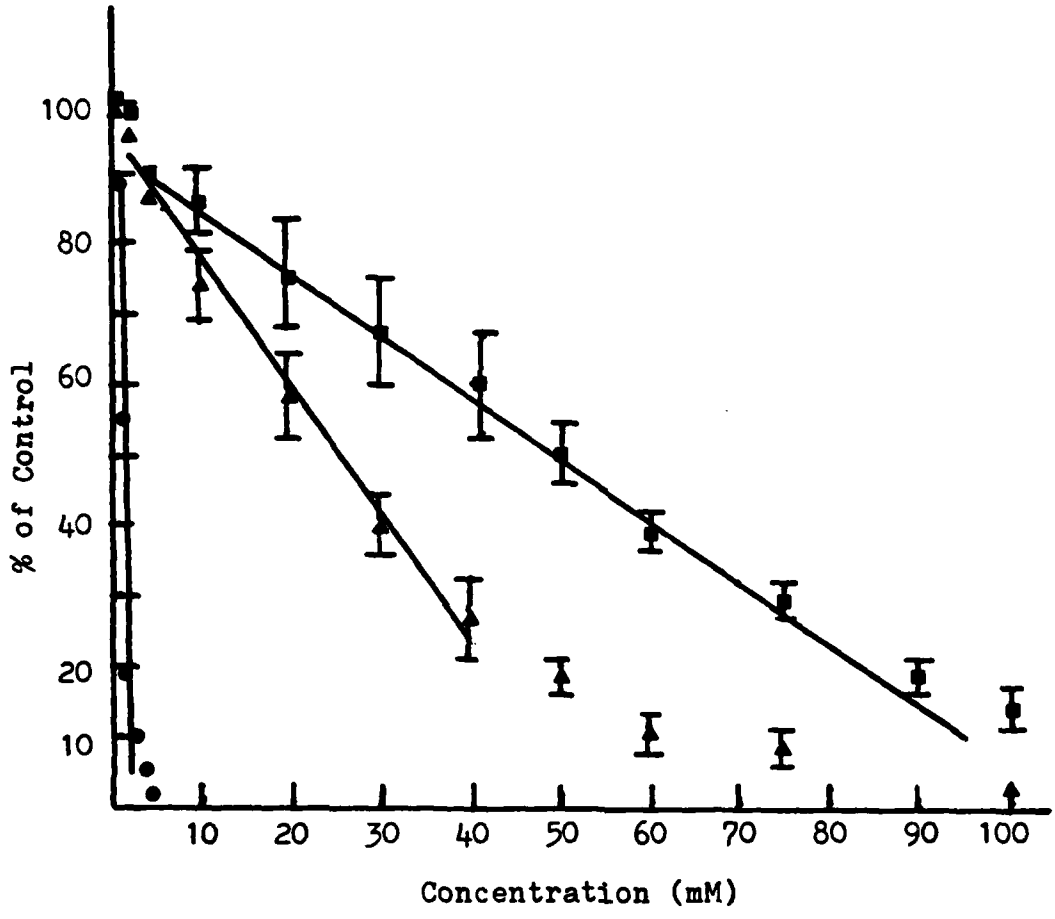



Figure 1 The effect of 48 hr exposure to acrylamide (●——●), N-methylacrylamide (▲——▲), or crotonamide (■——■) on the plating efficiency of log phase N1E-115 neuroblastoma cells (± S.E.).

When measuring AchE activity it is important to block the action of other esterases which may be present and may interfere with the reaction. There are other esterases present in neuroblastoma cells. Butyrylesterase is not present in significant amounts in cells of neuronal origin (Schubert et al, 1971); Prasad and Vernadakis, 1972), but cholinesterase and acetylesterase are present in measurable amounts in neuroblastoma cells (Blume et al, 1970). These pseudoesterases are selectively inhibited by iso-OMPA (El-Badawi and Schenk, 1967; Blume et al, 1970), which was added to all reaction mixtures. Controls run with Eel electric organ AchE, in the presence of iso-OMPA indicated only a 5% decrease in AchE activity.

Log phase cultures treated with AA showed a substantial decrease in AchE activity at concentrations above 0.5 mM. Control AchE activity in log phase cells was 82.1 nmol product formed/min/mg protein. The $LD_{50}$ by AA for AchE activity in log phase cells was 2.0 mM (37 nmol product/min/mg protein), with linear portion of the dose response curve showing a slope of -13 between doses of 0.5 mM AA and 4.0 mM AA.

Log phase cultures of NIE-115 neuroblastoma cells treated with NMA at doses of 10 to 30 mM were induced to increase their levels at AchE activity by as much as 56%, from 82.1 nmol of product formed/min/mg protein to 128 nmol/min/mg protein. Similar results were observed with 30 to 50 mM doses of CA (Fig. 2). Higher doses of both NMA and CA led to inhibition of AchE activity. N-methylacrylamide treated cultures had a dose response curve with a slope of -1.5, as compared to -13 for AA, in the linear response region from 30 to 100. The $ID_{50}$ for NMA was measured at a dose of 75 mM. Crotonamide-treated cultures showed a linear dose response from 40 to 100 mM with a slope of -1.4, which was similar to that measured for NMA, but much less than that

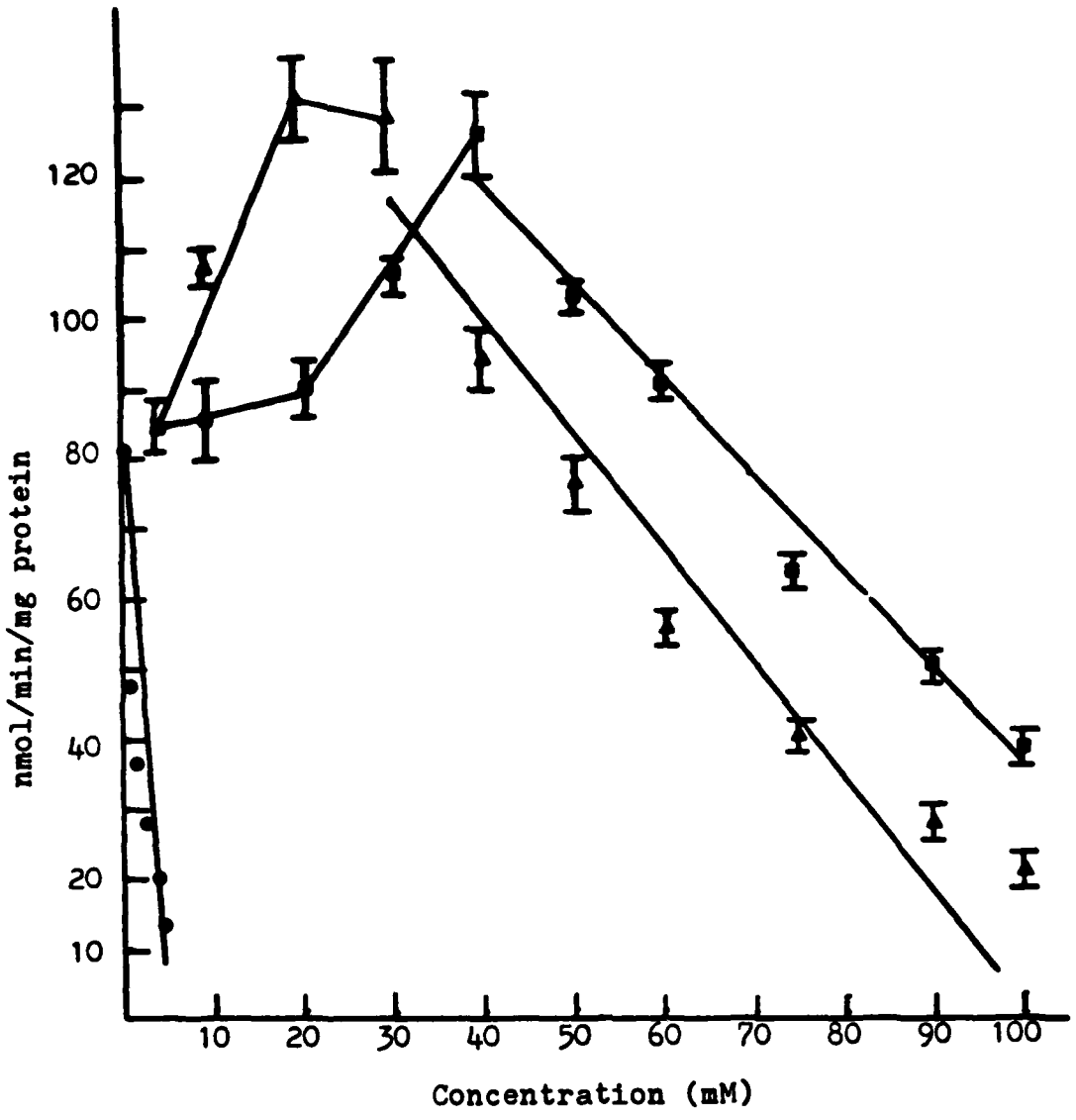



Figure 2 The effect of 48 hr exposure to acrylamide (●——●), N-methylacrylamide (▲——▲), or crotonamide (■——■) on the acetylcholinesterase activity of log phase N1E-115 neuroblastoma cells (± S.E.).

TABLE 3

THE EFFECT OF 48 HOUR ACRYLAMIDE (AA), N-METHYLACRYLAMIDE (NMA), OR CROTONAMIDE (CA) EXPOSURE ON THE MACROMOLECULAR CONTENT OF DIFFERENTIATED N1E-115 NEUROBLASTOMA CELLS

| Xenobiotic | Dose (mM) | µg Protein* | | µg RNA* | | µg DNA* | |
|---|---|---|---|---|---|---|---|
| | | $10^6$ cells | $10^6$ VC** | $10^6$ cells | $10^6$ VC** | $10^6$ cells | $10^6$ VC** |
| Control | - | 713 ± 15 | 713 ± 15 | 202 ± 6.4 | 202 ± 6.4 | 4.6 ± 0.3 | 4.6 ± 0.3 |
| AA | 0.01 | 708 ± 23 | 761 ± 25 | 193 ± 12 | 208 ± 13 | 4.9 ± 0.2 | 5.2 ± 0.2 |
| AA | 0.05 | 698 ± 21 | 793 ± 26 | 213 ± 10 | 242 ± 12 | 4.8 ± 0.3 | 5.5 ± 0.3 |
| AA | 0.10 | 718 ± 16 | 835 ± 22 | 198 ± 14 | 230 ± 17 | 4.8 ± 0.2 | 5.6 ± 0.3 |
| AA | 0.50 | 683 ± 20 | 1030 ± 30 | 213 ± 11 | 323 ± 16 | 5.3 ± 0.4 | 8.0 ± 0.6 |
| AA | 1.00 | 646 ± 26 | 1240 ± 50 | 191 ± 11 | 367 ± 19 | 4.1 ± 0.3 | 7.9 ± 0.6 |
| AA | 2.00 | 588 ± 17 | 2560 ± 67 | 194 ± 13 | 843 ± 40 | 5.4 ± 0.4 | 24 ± 2 |
| NMA | 5.0 | 718 ± 17 | 772 ± 19 | 220 ± 8.0 | 237 ± 9.2 | 4.9 ± 0.3 | 5.3 ± 0.4 |
| NMA | 10.0 | 711 ± 20 | 808 ± 22 | 205 ± 13 | 233 ± 14 | 5.0 ± 0.3 | 5.7 ± 0.4 |
| NMA | 20.0 | 681 ± 18 | 873 ± 22 | 200 ± 12 | 256 ± 15 | 4.9 ± 0.2 | 6.3 ± 0.3 |
| NMA | 30.0 | 677 ± 23 | 1170 ± 45 | 219 ± 7.9 | 378 ± 14 | 5.2 ± 0.3 | 9.0 ± 0.5 |
| NMA | 40.0 | 611 ± 19 | 1490 ± 40 | 217 ± 16 | 529 ± 36 | 5.6 ± 0.3 | 14 ± 0.7 |
| NMA | 50.0 | 303 ± 16 | 1317 ± 46 | 181 ± 8.9 | 787 ± 35 | 5.2 ± 0.3 | 23 ± 2 |
| CA | 5.0 | 691 ± 20 | 691 ± 20 | 173 ± 10 | 173 ± 10 | 4.1 ± 0.3 | 4.1 ± 0.3 |
| CA | 10.0 | 708 ± 18 | 730 ± 19 | 236 ± 16 | 242 ± 17 | 5.0 ± 0.3 | 5.2 ± 0.3 |
| CA | 20.0 | 701 ± 19 | 754 ± 21 | 188 ± 17 | 202 ± 18 | 4.9 ± 0.4 | 5.3 ± 0.4 |
| CA | 30.0 | 684 ± 22 | 834 ± 25 | 208 ± 16 | 254 ± 18 | 5.4 ± 0.3 | 6.6 ± 0.4 |
| CA | 40.0 | 667 ± 20 | 981 ± 30 | 196 ± 6.5 | 288 ± 10 | 5.4 ± 0.2 | 8.0 ± 0.3 |
| CA | 50.0 | 602 ± 24 | 1180 ± 47 | 178 ± 12 | 349 ± 24 | 5.4 ± 0.4 | 11 ± 0.8 |
| CA | 75.0 | 387 ± 16 | 992 ± 36 | 186 ± 10 | 477 ± 28 | 4.9 ± 0.2 | 12 ± 0.7 |

*± S.E.
** Viable Cells

TABLE 3

THE EFFECT OF 48 HOUR ACRYLAMIDE (AA), N-METHYLACRYLAMIDE (NMA), OR CROTONAMIDE (CA) EXPOSURE ON THE MACROMOLECULAR CONTENT OF DIFFERENTIATED N1E-115 NEUROBLASTOMA CELLS

| Xenobiotic | Dose (mM) | µg Protein* | | µg RNA* | | µg DNA* | |
|---|---|---|---|---|---|---|---|
| | | $10^6$ cells | $10^6$ VC** | $10^6$ cells | $10^6$ VC** | $10^6$ cells | $10^6$ VC** |
| Control | - | 713 ± 15 | 713 ± 15 | 202 ± 6.4 | 202 ± 6.4 | 4.6 ± 0.3 | 4.6 ± 0.3 |
| AA | 0.01 | 708 ± 23 | 761 ± 25 | 193 ± 12 | 208 ± 13 | 4.9 ± 0.2 | 5.2 ± 0.2 |
| AA | 0.05 | 698 ± 21 | 793 ± 26 | 213 ± 10 | 242 ± 12 | 4.8 ± 0.3 | 5.5 ± 0.3 |
| AA | 0.10 | 718 ± 16 | 835 ± 22 | 198 ± 14 | 230 ± 17 | 4.8 ± 0.2 | 5.6 ± 0.3 |
| AA | 0.50 | 683 ± 20 | 1030 ± 30 | 213 ± 11 | 323 ± 16 | 5.3 ± 0.4 | 8.0 ± 0.6 |
| AA | 1.00 | 646 ± 26 | 1240 ± 50 | 191 ± 11 | 367 ± 19 | 4.1 ± 0.3 | 7.9 ± 0.6 |
| AA | 2.00 | 588 ± 17 | 2560 ± 67 | 194 ± 13 | 843 ± 40 | 5.4 ± 0.4 | 24 ± 2 |
| NMA | 5.0 | 718 ± 17 | 772 ± 19 | 220 ± 8.0 | 237 ± 9.2 | 4.9 ± 0.3 | 5.3 ± 0.4 |
| NMA | 10.0 | 711 ± 20 | 808 ± 22 | 205 ± 13 | 233 ± 14 | 5.0 ± 0.3 | 5.7 ± 0.4 |
| NMA | 20.0 | 681 ± 18 | 873 ± 22 | 200 ± 12 | 256 ± 15 | 4.9 ± 0.2 | 6.3 ± 0.3 |
| NMA | 30.0 | 677 ± 23 | 1170 ± 45 | 219 ± 7.9 | 378 ± 14 | 5.2 ± 0.3 | 9.0 ± 0.5 |
| NMA | 40.0 | 611 ± 19 | 1490 ± 40 | 217 ± 16 | 529 ± 36 | 5.6 ± 0.3 | 14 ± 0.7 |
| NMA | 50.0 | 303 ± 16 | 1317 ± 46 | 181 ± 8.9 | 787 ± 35 | 5.2 ± 0.3 | 23 ± 2 |
| CA | 5.0 | 691 ± 20 | 691 ± 20 | 173 ± 10 | 173 ± 10 | 4.1 ± 0.3 | 4.1 ± 0.3 |
| CA | 10.0 | 708 ± 18 | 730 ± 19 | 236 ± 16 | 242 ± 17 | 5.0 ± 0.3 | 5.2 ± 0.3 |
| CA | 20.0 | 701 ± 19 | 754 ± 21 | 188 ± 17 | 202 ± 18 | 4.9 ± 0.4 | 5.3 ± 0.4 |
| CA | 30.0 | 684 ± 22 | 834 ± 25 | 208 ± 16 | 254 ± 18 | 5.4 ± 0.3 | 6.6 ± 0.4 |
| CA | 40.0 | 667 ± 20 | 981 ± 30 | 196 ± 6.5 | 288 ± 10 | 5.4 ± 0.2 | 8.0 ± 0.3 |
| CA | 50.0 | 602 ± 24 | 1180 ± 47 | 178 ± 12 | 349 ± 24 | 5.4 ± 0.4 | 11 ± 0.8 |
| CA | 75.0 | 387 ± 16 | 992 ± 36 | 186 ± 10 | 477 ± 28 | 4.9 ± 0.2 | 12 ± 0.7 |

*± S.E.
** Viable Cells

TABLE 4

THE EFFECT OF 48HOUR ACRYLAMIDE (AA), N-METHYLACRYLAMIDE (NMA), OR CROTONAMIDE (CA) EXPOSURE ON THE MACROMOLECULAR SYNTHESIS RATES OF LOG PHASE N1E-115 NEUROBLASTOMA CELLS

| Xenobiotic | Dose (mM) | CPM* / µg Protein | CPM* / µg RNA | CPM* / µg DNA |
|---|---|---|---|---|
| Control | - | 117 ± 11 | 2160 ± 50.8 | 2480 ± 101 |
| AA | 0.05 | 105 ± 15 | 1730 ± 97.0 | 2310 ± 98.5 |
| AA | 0.10 | 113 ± 16 | 2310 ± 114 | 2060 ± 76.9 |
| AA | 0.50 | 117 ± 22 | 1830 ± 97.5 | 1810 ± 112 |
| AA | 1.00 | 100 ± 13 | 1820 ± 121 | 2130 ± 95.9 |
| AA | 2.00 | 85.3 ± 18 | 1580 ± 79.3 | 1320 ± 89.3 |
| AA | 3.00 | 57.5 ± 17 | 859 ± 66 | 66.8 ± 15 |
| NMA | 5.0 | 341 ± 32 | 2200 ± 107 | 1910 ± 99.3 |
| NMA | 10.0 | 376 ± 23 | 2430 ± 135 | 1200 ± 77.2 |
| NMA | 20.0 | 276 ± 15 | 2680 ± 85.9 | 976 ± 56 |
| NMA | 30.0 | 231 ± 18 | 2110 ± 117 | 87.9 ± 12 |
| NMA | 40.0 | 153 ± 7.4 | 1010 ± 66.4 | 42.3 ± 4.8 |
| NMA | 50.0 | 70.2 ± 3.2 | 496 ± 12 | 70.7 ± 5.8 |
| CA | 10.0 | 133 ± 22 | 1960 ± 114 | 2320 ± 114 |
| CA | 20.0 | 173 ± 19 | 2280 ± 125 | 1990 ± 142 |
| CA | 30.0 | 236 ± 30 | 2670 ± 111 | 1770 ± 130 |
| CA | 40.0 | 244 ± 10 | 2430 ± 88.4 | 1350 ± 77.3 |
| CA | 50.0 | 203 ± 12 | 1840 ± 73.7 | 863 ± 23 |
| CA | 75.0 | 106 ± 7.4 | 321 ± 22 | 66.8 ± 7.5 |

*± S.E.

TABLE 5

THE EFFECT OF 48 HOUR ACRYLAMIDE (AA), N-METHYLACRYLAMIDE (NMA), OR CROTONAMIDE (CA) EXPOSURE ON THE MACROMOLECULAR SYNTHESIS RATES OF DIFFERENTIATED N1E-115 NEUROBLASTOMA CELLS

| Xenobiotic | Dose (mM) | CPM* / µg Protein | CPM* / µg RNA | CPM* / µg DNA |
|---|---|---|---|---|
| Control | - | 76.0 ± 7.3 | 828 ± 23 | 131 ± 14 |
| AA | 0.01 | 82.7 ± 2.5 | 886 ± 22 | 183 ± 14 |
| AA | 0.05 | 80.6 ± 5.6 | 751 ± 15 | 112 ± 10 |
| AA | 0.10 | 79.6 ± 3.5 | 689 ± 15 | 197 ± 11 |
| AA | 0.50 | 82.9 ± 5.4 | 677 ± 12 | 155 ± 15 |
| AA | 1.00 | 69.0 ± 0.75 | 625 ± 9.6 | 146 ± 14 |
| AA | 2.00 | 67.1 ± 2.6 | 609 ± 10 | 53.3 ± 8.5 |
| NMA | 5.0 | 89.4 ± 3.2 | 1170 ± 16 | 104 ± 4.3 |
| NMA | 10.0 | 87.5 ± 6.0 | 1100 ± 40 | 89.1 ± 5.4 |
| NMA | 20.0 | 82.8 ± 4.8 | 814 ± 23 | 44.0 ± 0.42 |
| NMA | 30.0 | 78.3 ± 9.2 | 601 ± 11 | 58.7 ± 0.69 |
| NMA | 40.0 | 62.1 ± 0.92 | 312 ± 4.9 | 0 |
| NMA | 50.0 | 33.8 ± 1.1 | 7.02 ± 0.33 | 0 |
| CA | 5.0 | 88.2 ± 4.9 | 715 ± 18 | 82.2 ± 8.2 |
| CA | 10.0 | 79.3 ± 3.2 | 730 ± 10 | 136 ± 7.4 |
| CA | 20.0 | 81.3 ± 2.2 | 619 ± 13 | 122 ± 2.4 |
| CA | 30.0 | 74.7 ± 2.9 | 820 ± 7.5 | 117 ± 10 |
| CA | 40.0 | 78.3 ± 3.6 | 433 ± 5.9 | 66.1 ± 0.55 |
| CA | 50.0 | 66.3 ± 0.94 | 265 ± 2.6 | 78.2 ± 1.2 |
| CA | 75.0 | 44.7 ± 1.3 | 97.8 ± 0.83 | 49.7 ± 2.3 |

* ± S.E.

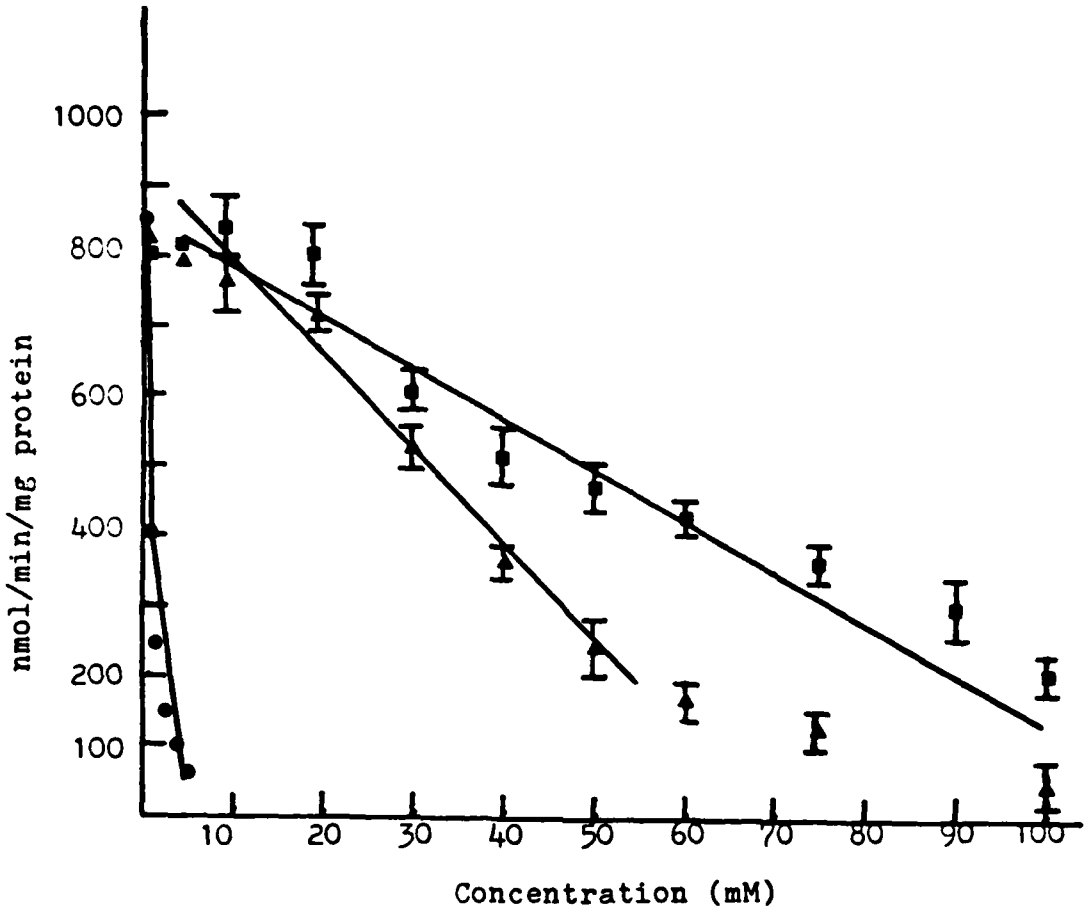



Figure 3 The effect of 48 hr exposure to acrylamide (●——●), N-methylacrylamide (▲——▲), or crotonamide (■——■) on the acetylcholinesterase activity of differentiated N1E-115 neuroblastoma cells (± S.E.).

observed for AA. The $ID_{50}$ for CA dosed cultures was at 100 mM.

Differentiated cells were more sensitive to the effects of the xenobiotics, and had steeper dose response curves (Fig. 3). The dose response curve for AA was very steep from 0.01 mM or 0.5 mM, with a slope of -100. The dose response curve for AA became less steep from 0.5 to 5.0 mM with a slope of -8.3. The $ID_{50}$ for AchE activity in AA treated differentiated cells was at a dose of 0.5 mM as compared to 2.0 mM for log phase cells. N-methylacrylamide-treated differentiated cells ahd a $ID_{50}$ at 35 mM with a slope of -1.6, in contrast to CA-dosed cells which had an $ID_{50}$ of 60 mM and a slope of -0.59.

2.2.9. Neuron Specific Enolase (NSE) and Acrylamide Toxicity:

Recent reports implicate NSE as the possible site for AA neurotoxicity (Howland et al, 1980a). This neuron specific form of enolase is the only active enolase located in functioning neurons (Marangos and Schmechel, 1980). NIE-115 neuroblastoma cells can be induced to synthesize NSE by treating the cells with dibutryl cAMP or by allowing the cells to grow to stationary phase (Marangos et al, 1978; Le Gault-Demare et al, 1980). This enzyme is an important glycolytic enzyme which catalyzes the reaction of 2-phosphoglycerate to phosphoenolpyruvate. Therefore, any substance which inhibits this enzyme could cause an energy depletion in the cell.

The total enolase activity of log phase cells was studied and showed no inhibition by doses of AA, NMA, and CA over the entire range tested (data not graphed). Log phase cultures had a total enolase activity of 1.9 micromoles of product formed/min/mg/ protein.

Figure 4 shows the effect of doses of AA, NMA and CA on the NSE activity of differentiated cells. The $ID_{50}$ dose was o.5 mM AA. The dose response

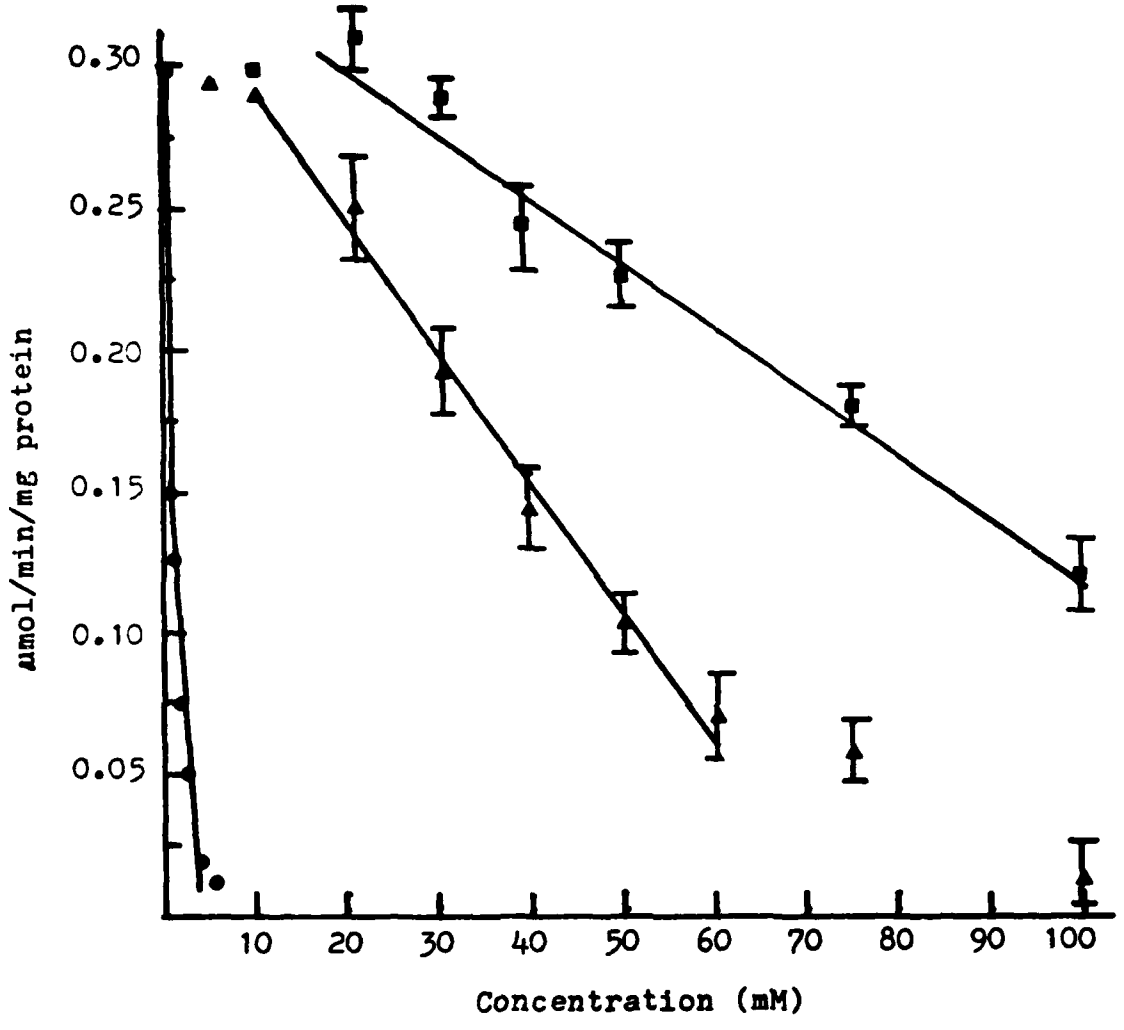



Figure 4 The effect of 48 hr exposure to acrylamide (●——●), N-methylacrylamide (▲——▲), or crotonamide (■——■) on the neuron specific enolase activity of differentiated N1E-115 neuroblastoma cells (± S.E.).

curve had a slope of -103 in the linear region which extended from a dose of 0.05 mM AA to the $ID_{50}$ dose of 0.5 mM AA. The dose response curve became less steep from 0.5 mM AA to 4.0 mM AA where the slope was -12.

N-methylacrylamide inhibited NSE activity to a lesser extent than AA. The $ID_{50}$ dose was 50 mM NMA. A shoulder was evident from 1.0 to 10 mM NMA with the linear part of the dose response curve extending from 10 to 60 mM NMA. This linear region had a slope of -1.5.

Crotonamide also was less reactive in inhibiting the activity of NSE. One hundred and sixty-fold higher doses of CA were necessary to reach the $ID_{50}$ (80 mM). The slope of the CA dose response curve was -0.66 in the linear response region from doses of 20 to 100 mM. This slope was only half that measured for NMA. A shoulder was noted from doses of 1.0 to 20 mM CA.

The total enolase activity of differentiated NIE-115 neuroblastoma cells was not greatly affected by either AA, NMA, or CA.

2.2.10. Effect of Organophosphates on Neurotoxic Esterase (NTE) Activity in Neuroblastoma Cells:

The neuroblastoma cell cultures were found to have levels of NTE comparable to hen spinal cord or brain. Six mg of cellular material was required to perform the NTE assays while Johnson reports 6 mg of hen brain is required. Using phenyl valerate as substrate and homogenates of non-differentiated neuroblastoma cells, NTE was found to comprise 26% of the total esterase activity. This figure is 47% of the paraoxon-resistant activity. Due to the lack of commercially available phenyl valerate we sought out a replacement substrate which would be readily available and still serve as a sensitive and specific substitute for NTE. Phenyl 4-chlorobutyrate from Aldrich Chemical Co., was chosen due to its structural similarity to phenyl valerate. In an experiment identical to that carried out with phenyl valerate, phenyl 4-chlorobutyrate yielded very similar results. NTE was found to comprise

20% of the total esterase activity representing 37% of the paraoxon-resistant esterase activity. Using phenyl valerate and hen brain homogenate Johnson (1980) found that NTE comprises 16% of the total esterase activity or 64% of the paraoxon-resistant fraction. Dose response curves of all OPs tested were identical with both substrates (Fig. 5).

Using homogenates of 74-hour differentiated cultures, NTE was found to comprise 35% of the total esterase activity amounting to 58% of the paraoxon-resistant fraction. This represents a 75% increase of NTE in differentiated cells over that found in non-differentiated cells. The largest NTE fraction was observed when intact cells were used and they were treated in situ with paraoxon and mipafox (Fig. 6). NTE was found to account for 43% of the total esterase, which represents 66% of the paraoxon-resistant fraction. The OPs which were studied inhibited NTE to the extent specified: leptophos 26%; EPN 14%; OMPA 21%; and DFP 77% (Fig. 7). A value of 70% inhibition or greater is indicative of a compound causing OPIDN (Johnson, 1974). DFP is the most potent esterase inhibitor in animals (Johnson, 1980) and ranked as such in our test system. EPN and leptophos have been reported to induce OPIDN in animals but came up negative in this assay system (Abou-Donia, 1979). Leptophos must be converted metabolically to leptophos-oxon before it exerts its neurotoxic effects (Abou-Donia, 1981). In order to determine whether or not the cell culture system's lack of responsiveness to leptophos could stem from its inability to activate leptophos, a side by side dose response study of leptophos and leptophos-oxon was performed with intact cells. At concentrations of 50, 100, and 200 μM, leptophos inhibited NTE by 10, 17, and 34%, respectively, while leptophos-oxon inhibited 97, 100, and 100%. This same situation is true for EPN which also must be metabolically activated to the

FIG. 5 DOSE RESPONSE CURVES OF SIX ORGANOPHOSPHATES ON TOTAL ESTERASE ACTIVITY OF NIE-115 HOMOGENATES WITH PHENYL 4-CHLOROBUTYRATE SUBSTRATE

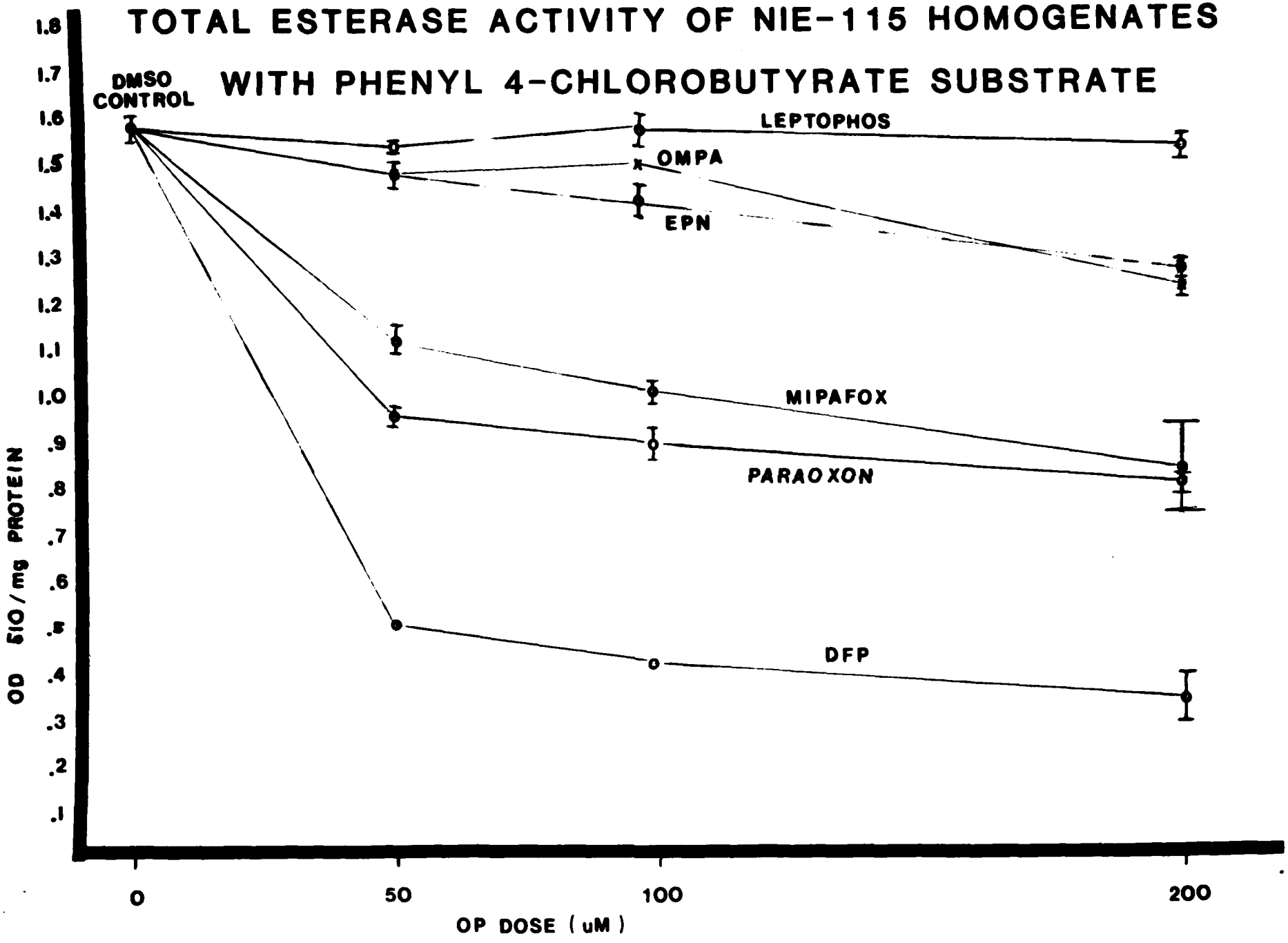


FIG. 6 **Esterase fractions from in situ treatment of nondifferentiated NIE-115 using Phenyl-4-chlorobutyrate substrate**

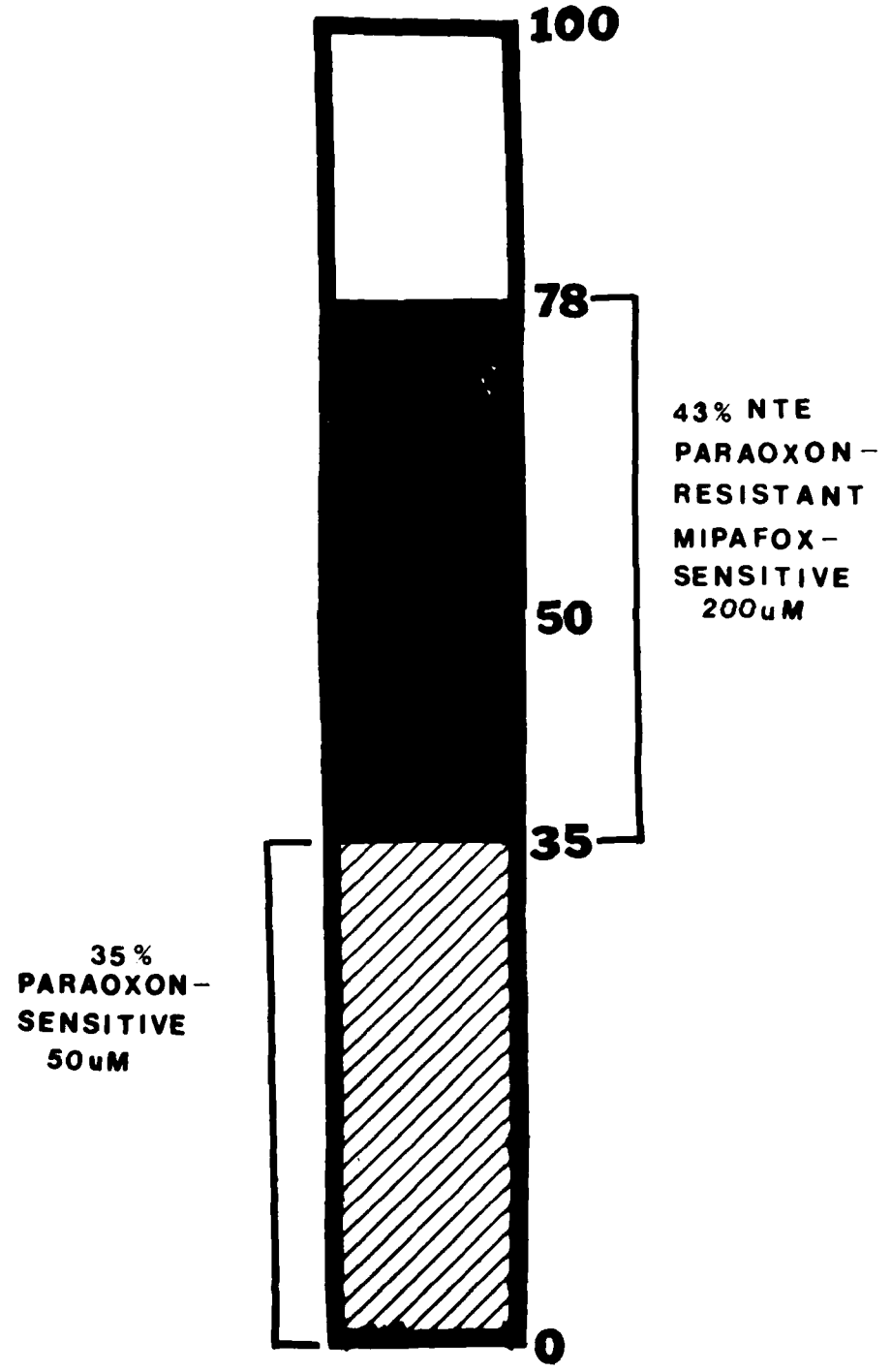
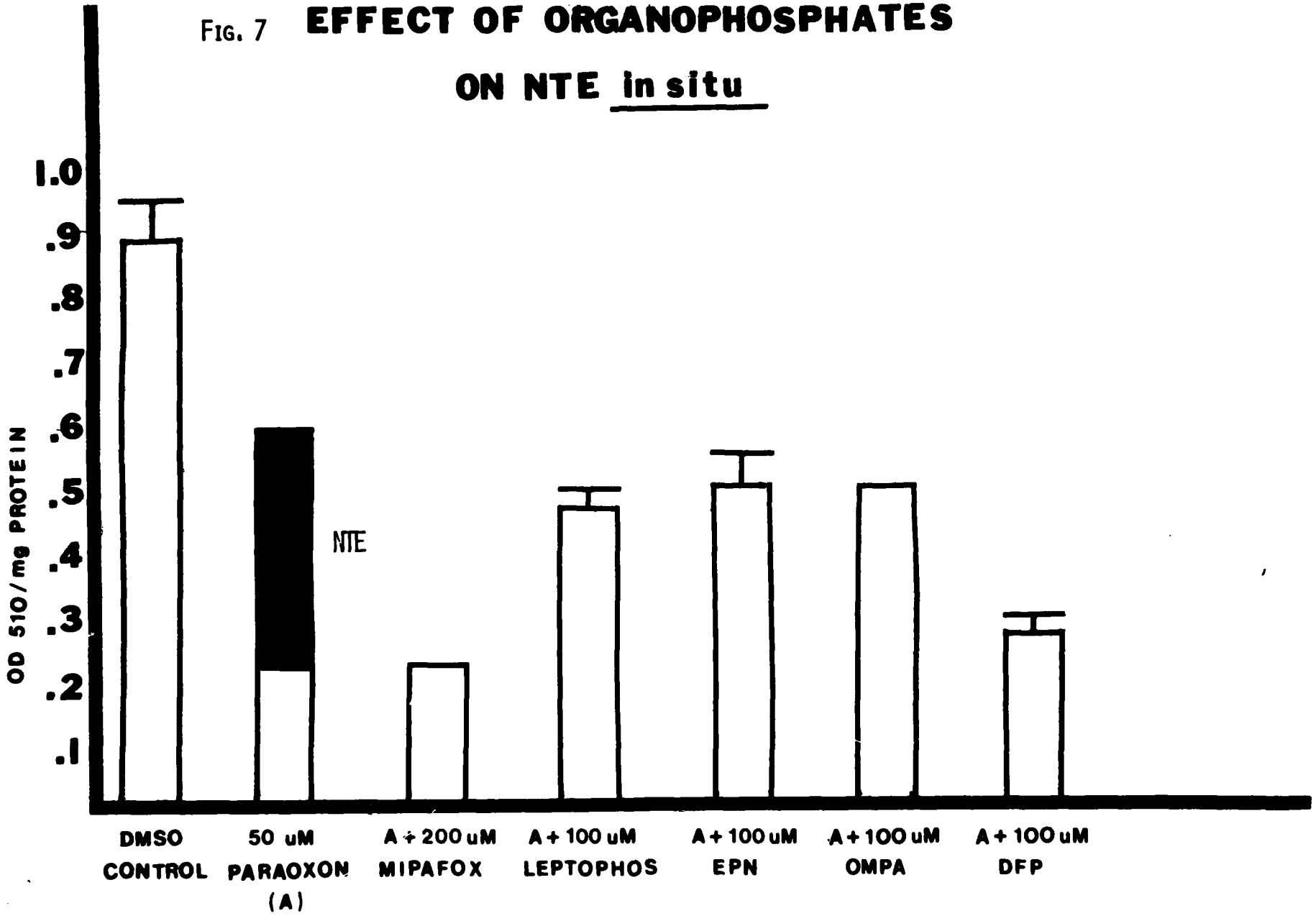


FIG. 7 **EFFECT OF ORGANOPHOSPHATES ON NTE in situ**



EPN-oxon formed. All known neurotoxic compounds tested including DFP and leptophos-oxon yielded an NTE inhibition of well over the minimum 70% figure. No false negatives were obtained when the OPs used did not require metabolic activation. These data stress the importance of coupling a metabolic activation system with the target cells grown in culture. The development of a system suitable for testing indirect acting (require activation) OPs is an objective of the ongoing research.

2.2.11. An In Vitro Alternative for Testing the Effects of Organophosphates on Neurotoxic Esterase Activity:

The data obtained to date strongly suggests that the system we have developed mirrors the in vivo hen brain assay system used for the study of OPs on NTE. We propose that it be considered as an in vitro alternative should the validation experiments present no surprises.

Presently, potentially useful OPs are screened for delayed neurotoxic effects by administration of the OPs to hens after which they are observed for ataxia and paralysis and their brains are assayed for NTE inhibition. Johnson (1977) has also demonstrated that brain homogenates rather than whole animals could be used to test the OPs for NTE inhibition. We have adopted both of these in vivo approaches for in vitro testing. Intact cultures of differentiated neuroblastoma cells (NIE-115) are used in place of whole animals and cell culture homogenates are used in place of brain homgenates. NTE represents about 75% of the total esterase activity found in cell homogenates using phenyl valerate as a substrate. The non-neurotoxic OP paraoxon is used to inhibit similar but irrelevant esterase activity which represnts about 50% of the total in both systems. The neurotoxic OP mipafox inhibited an additional 25% of the total activity in cell homogenates which represents

the NTE fraction. Two other OPs, leptophos and EPN were found to cause an additional 10% inhibition beyond the paraoxon sensitive portion in both systems. Six mg of cell pellet are required to run this assay. These figures correspond well to known values obtained from the hen system. This in vitro system could prove to be a rapid and relatively inexpensive tool for the screening of potentially useful OPs prior to full scale use as well as reducing the need to test with animals.

An in vitro alternative is being proposed for several reasons. These include problems associated with (1) obtaining reproducible results with the hen assay, (2) economic factors, and (3) the animal rights movement. There are over 500 species of chickens with such genetic diversity that enzyme levels and responses to xenobiotics vary greatly. A supply of genetically non-variant and physically healthy hens is not always available either. Many environmental factors may alter the outcome of toxicological evaluations. Some of these are temperature, feed, photoperiod, type of housing, pen mates, animal handlers, sounds and disease. A lack of proper control over these factors may cause stress in the animal thereby affecting clinical signs as well as biochemical end-points. High stress diseases in the hen include viral infection, Mareks tumor, and mycoplasma while bacterial infections and mites induce less stress. Whether or not a hen is accustomed to human handling and interaction and thereby "socialized" is another often overlooked factor in toxicological studies involving the hen. Non-socialized hens tend to exhibit erratic response to chemical exposure. A hen's response to drugs depends on genetic heritage and the interaction of all previous events. In addition, commercial exposures to a wide variety of agents which may induce lesions within the nervous system. Some of these agents include infectious

organisms, attenuated live virus vaccines, chemcial-toxicants, and nutritional deficiences. Housing animals in wire mesh cages where gripping is constantly required has been reported to produce peripheral nerve lesions (Cotard-Bartley, 1981). Caring for a hen population requires a considerable investment in time, space, and financial resources as well as requiring considerable knowledge.

While a cost analysis of cell culture vs hen assay is premature, it appears that except for the special skill required for reproducible cell culture work, the space, veterinarian and handling costs for hens would be greater than the space, equipment and culture costs for cells.

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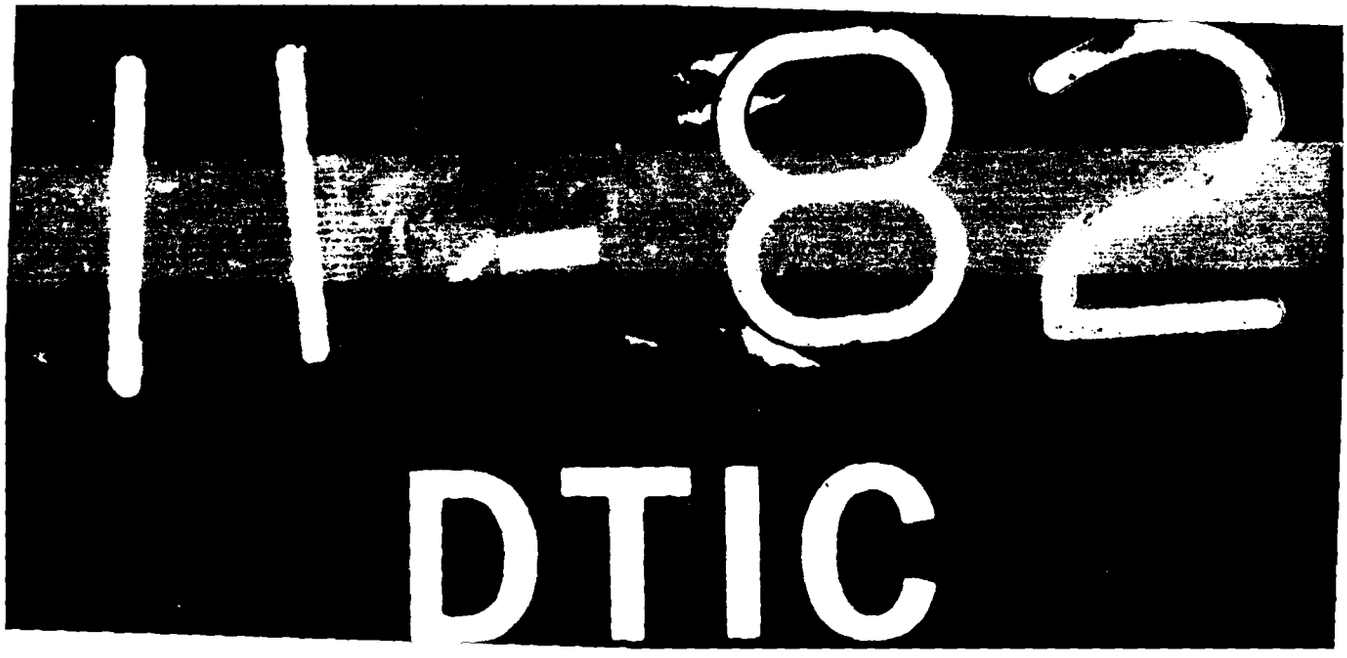
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